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The Second International Standard for Prolactin

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In 1953 the WHO Expert Committee on Biological Standardization asked the National Institute for Medical Research, London, to collect and study suitable material to replace the First International Standard for Prolactin, stocks of which were running low. This paper reports the nature and handling of the proposed replacement material, its potency as determined by comparison with the First International Standard by international collaborative assay, and its establishment as the Second International Standard for Prolactin, with a defined potency of 22 International Units per mg. The International Unit of Prolactin is defined as the activity contained in 0.04545 mg of the Second International Standard.

THE FIRST INTERNATIONAL STANDARD FOR PROLACTIN

The First International Standard for Prolactin, established in 1939 (League of Nations, 1939) consisted of 109 g of prolactin obtained as batches from nine different sources. The species of origin of the nine samples are unknown. The material was blended, dried over P_2O_5 and compressed into tablets of approximately 10 mg. A total of 932 ampoules was prepared, each containing 10 tablets. After comparison by assay, using the pigeon-crop method, with the preparation supplied by Dr Riddle of the Carnegie Institute of Washington, Cold Spring Harbor, N. Y., USA, which had been used widely as an unofficial standard, each sample was found to contain at least five " Riddle units " per mg. The potency of the standard itself was defined as 10 IU/mg. There was no estimation of the amount of contaminating hormones, although subsequent examination indicated that it contained about 0.07 IU of thyrotrophin hormone (TSH) activity per mg when assayed by ^{131}I thyroid depletion in chicks (Bates & Cornfield, 1957). The tablets, although convenient for weighing, proved difficult to dissolve and it was sometimes necessary to grind them in dilute alkali to effect complete solution.

By 1952 stocks of this standard were running low. In 1953 the WHO Expert Committee on Biological Standardization (1954) asked the National Institute for Medical Research, London, to obtain material suitable for the replacement of the First Standard. For the next three years, however, no satisfactory material was forthcoming.

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PROPOSED REPLACEMENT MATERIAL FOR THE INTERNATIONAL STANDARD

In 1956 the Armour Company, Kankakee, Ill., USA, generously made available to WHO 50 g of a freeze-dried preparation. The material is a freeze-dried powder (part of Lot D 14083-2B), prepared from sheep pituitary glands by extraction with acid acetone and purified by means of oxycellulose, sodium chloride and ammonium sulfate fractionation. It arrived at the Department of Biological Standards, National Institute for Medical Research, in a single screw-capped bottle in September 1956, and was kept with silica gel in a sealed envelope in the dark at −10°C until it was subjected to preliminary examination. The potency of the original powder was described by the Armour Laboratories as 28.9 ± 4.8 IU/mg, and the concentration of thyrotrophic oxytocic vasopressor and corticotrophic activities was less than 0.05 IU/mg in each case.

For six weeks prior to distribution in December 1957, the contents of the bottle were exposed to P_2O_5 in an evacuated desiccator. A portion of the material was then weighed and dissolved in distilled water to give a final concentration of 10.0 mg/ml. The solution was dispensed in 1-ml amounts into 2330 ampoules during one day, and freeze-dried as one batch. The ampoules were then placed over fresh P_2O_5 in evacuated desiccators for two weeks; after constricting they were dried for a further week, filled three times with dry nitrogen and sealed. They have since been stored in the dark at −10°C.

Each of the ampoules contains 10 ± 0.5 mg of the dried powder. The absolute moisture content has not yet been determined but is considered to be less than 2%. The powder is hygroscopic and care must be taken if it is weighed. For most assay purposes it could be assumed, without further weighing, that an ampoule contains 10 mg, since the variation in estimated potency is likely to exceed the (maximum) 5% filling error. The contents of the ampoule dissolve very readily in water or saline, but to ensure complete solution it is suggested that the water or saline is adjusted to pH 8 or 9.

THE COLLABORATIVE ASSAY

In June 1958 15 laboratories in 10 different countries were invited to take part in the collaborative assay of the proposed Second Standard; of these, nine laboratories in five countries agreed. Participants were asked to assay the material by any methods in which they had experience and confidence. In view of the anticipated difficulty in assembling a sufficient number of results, participants were asked to include assays involving the measurement of the increase in crop-weight in pigeons after injection of the dose systemically and/or locally. Participants were also invited to analyse the material for contaminating hormones. Details of methods, together with the assay data, were returned to the Department of Biological Standards, where the statistical analysis was carried out.

Results of the collaborative assay

Details of 29 separate assays were received from the nine participating laboratories which are listed in the Annex. Throughout this report the laboratories are referred to by arbitrary numbers which do not necessarily correspond to the order in which the laboratories are listed in the Annex.

Laboratories 1 to 8 all used systemic methods in pigeons, measuring the increase in crop-weight as response. The assays varied in design (Tables 1 and 2) but the method used by Laboratories 1, 2 and 4 was substantially that of Bates & Riddle (1941) while Laboratories 5, 6, 7 and 8 used the method of Folley et al. (1940) but with variations from the six daily injections of 1.0 ml as originally described. Laboratory 3 administered prolactin by the subcutaneous route in two assays and by intramuscular injection in another two assays. White Carneaux pigeons were used as the test animal by Laboratories 1, 3 and 6, while Laboratory 4 used Swedish and Danish strains. The remainder were common pigeons of mixed origin.

Laboratory 9 estimated the activity of the new standard by measurement of the luteotrophic activity in hypophysectomized female rats of the Long-Evans strain (assay 27) and also by a local intradermal method in White King squabs (assays 28 and 29). About 100 rat and 300 pigeon responses were recorded in these tests.

Laboratory 1 examined the preparations under consideration for contaminating hormones and

TABLE 1

ASSAY DESIGN (SYSTEMIC METHODS)

Lab. No.	Assay No.	No. of doses [a]		Total No. of pigeons	Final weight of pigeons (range in g)
		S	U		
1	1	3	3	30	402-535
	2	3	3	30	430-581
2	3	3	3	120	220-456
3	4	3	3	54	Not recorded
	5	3	3	54	Not recorded
	6	2	2	44	330-570
	7	2	2	40	369-521
4	8	3	3	30	155-360
	9	3	3	30	230-435
5	10	4	4	40	343-475
	11	3	3	50	299-452
	12	2	2	44	256-448
	13	2	2	48	273-484
	14	2	2	23	300-404
	15	2	2	23	306-417
	16	2	2	24	282-456
	17	2	2	24	282-413
	18	2	2	24	256-391
	19	2	2	24	252-426
	20	2	2	24	254-398
	21	2	2	24	218-418
	22	2	2	24	252-350
	23	2	2	24	222-341
6	24	4	4	120	248-520
7	25	2	2	64	231-391
8	26	3	3	84	245-496

[a] S = First International Standard (Standard). U = Second International Standard (Unknown).

TABLE 2
DOSING SCHEDULE (SYSTEMIC METHODS)

Lab. No.	Assay No.	Total doses [a] (range in mg) S	U	Daily volume injected (ml)	No. of injections	Route of injections
1	1	1-4	0.5-2	0.5	4	Intramuscular
	2	1-4	0.5-2	0.5	4	"
2	3	0.25-4	0.06-1	0.5	4	Intramuscular
3	4	1-6	0.4-2.4	0.5	4	Intramuscular
	5	1-6	0.5-3	0.5	4	"
	6	0.6-2.4	0.3-1.2	0.5	4	Subcutaneous
	7	0.6-2.4	0.3-1.2	0.5	4	"
4	8	0.6-2.4	0.2-0.8	0.2	4	Intramuscular
	9	1.2-4.8	0.4-1.6	0.2	4	"
5	10	0.25-6.75	0.13-3.4	1	6	Subcutaneous
	11	0.6-2.4	0.2-0.8	1	6	"
	12	0.6-2.4	0.25-1	1	6	"
	13	0.6-2.6	0.23-0.93	1	7	"
	14	0.4-1.7	0.17-0.67	1	4	"
	15	0.6-2.5	0.25-1	1	6	"
	16	0.4-1.6	0.17-0.67	1	4	"
	17	0.6-2.4	0.25-1	1	6	"
	18	0.4-1.6	0.17-0.67	1	4	"
	19	0.6-2.4	0.25-1	1	6	"
	20	0.4-1.6	0.17-0.67	1	4	"
	21	0.6-2.4	0.25-1	1	6	"
	22	0.4-1.7	0.17-0.67	1	4	"
	23	0.6-2.5	0.25-1	1	6	"
6	24	0.25-1	0.13-0.5	0.5	4	Subcutaneous
7	25	0.5-1.6	0.3-0.9	1	4	Subcutaneous
8	26	0.6-1.35	0.3-0.7	1	6	Subcutaneous

[a] S = First International Standard (Standard). U = Second International Standard (Unknown).

estimated the TSH content of the International Standard as 0.07 USP u/mg and of the new standard as <0.001 USP u/mg. These values were estimated by ^{131}I depletion in the baby chick (Bates & Cornfield, 1957).

It was also stated that electrophoresis on starch gel revealed several small components.

Statistical analysis of the results of the collaborative assay

According to the literature there may be some advantage in using crop-weight expressed as a proportion of body-weight as the response metameter in pigeon assays.

Riddle et al. (1933) found that the weight of crop gland was directly proportional to body-weight in groups of mixed pigeons but that there was no correlation within any particular race. Bates & Riddle (1941) expressed the opinion that the " correction of crop sac weights for body weight hardly seems justified when the pigeons are of the same breed and age " but that such a correction would be necessary when mixed groups of pigeons are used. Folley et al. (1940) also found a correlation between crop-weight and body-weight in groups of mixed pigeons and obtained narrower limits of error by using relative crop-weight as metameter than by using the uncorrected crop-weight.

In the present study the assay with the largest number of pigeons at each dose level (assay 3) was examined for this correlation. A positive regression of crop-weight on body-weight was found at each dose level and all the fitted lines intersected the axes near the origin, indicating a direct relationship between the two variables. As further evidence in support of this relationship, the first four assays which were received (assays 10, 11, 12 and 13) were analysed using:

(*a*) crop-weight as response;

(*b*) crop-weight expressed as a percentage of final body-weight; and

(*c*) crop-weight with co-variance for body-weight.

The potencies of the proposed standard in terms of the First International Standard, which were estimated by these three methods, are given in Table 3 together with their associated weights, which were calculated as the reciprocals of the variances of the log potencies.

It was concluded that method (*b*) led to slightly narrower limits of error of the potency estimates while the lengthier co-variance analysis appeared to add nothing useful to the interpretation of the data. With the exception of assays 4 and 5, assays 1-26 have therefore been analysed by the standard method for parallel line assays, using relative crop-weight as response metameter. Since body-weights were not recorded for assays 4 and 5 the unadjusted crop-weight was used in the calculations.

For assays 27, 28 and 29 the response was measured quantally and the analysis was done by the standard probit method.

Each assay was examined for parallelism of the log-dose/response lines, and in only one assay (No. 6) was the term for parallelism in the analysis of variance significantly greater than the error variance.

TABLE 3
COMPARISON OF DIFFERENT METHODS OF ANALYSIS

Assay No.	Potency (IU/mg)	Weight (I/V)
	Crop-weight	
10	41.7	80
11	20.5	85
12	33.7	76
13	32.2	90
	Crop-weight as % of body-weight	
10	37.8	104
11	20.9	112
12	32.5	98
13	30.2	94
	Crop-weight with covariance for body-weight	
10	39.0	89
11	20.4	93
12	31.1	80
13	27.6	116

Examination of the slopes of the log-dose/response lines for each preparation revealed no systematic non-parallelism between preparations (Table 4).

For the assays where three or more doses of each preparation had been used the results were also examined for curvature of the log-dose/response lines, but no significant deviations from linearity were found in the analyses of variance. Apart from Laboratory 7, where the response lines were examined separately before the materials were assayed, each laboratory provided some tests using at least three dilutions of each preparation.

Since there was no evidence of statistical invalidity, a potency for the proposed standard in terms of the existing standard was estimated from each assay (Table 4). Assay 6, which had shown a significant departure from parallelism at the 5% level, was included with a reduced weight which was calculated by increasing the error variance to the point where it was not significantly less than the term for parallelism.

In the distribution of log potencies (see the figure) the height of each block is proportional to the statistical weight associated with the potency it represents.

TABLE 4

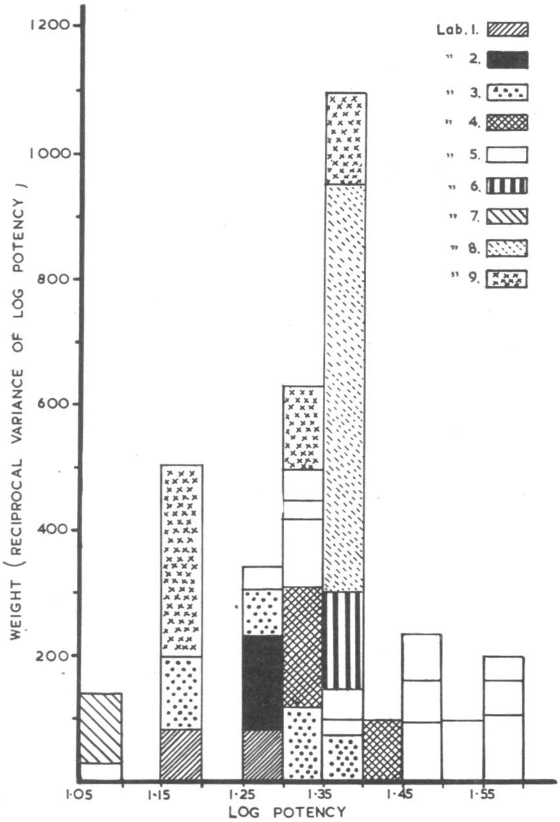
POTENCIES ESTIMATED FROM INDIVIDUAL ASSAYS

Lab. No.	Assay No.	Slopes of log-dose/ response lines		Potency (IU/mg)	Weight (I/V)	χ^2 [a]
		1st Int. Standard	2nd Int. Standard			
1	1	0.68	0.44	19.7	82	0.2
	2	0.66	0.34	15.3	83	2.1
2	3	0.68	0.73	19.0	152	0.6
3	4 [b]	2.32	2.02	15.4	120	2.9
	5 [b]	2.60	1.29	19.1	72	0.3
	6	0.88	0.39	24.2	74	0.1
	7	0.61	0.36	20.0	118	0.2
4	8	0.91	0.92	27.7	96	1.0
	9	0.82	0.98	22.3	188	0.0
5	10	0.67	0.79	37.8	104	5.7
	11	0.73	0.50	20.9	112	0.1
	12	0.47	0.48	32.5	98	2.8
	13	0.92	0.96	30.2	94	1.8
	14	0.47	0.50	28.6	66	0.9
	15	0.41	0.56	24.1	25	0.0
	16	0.39	0.59	24.9	46	0.1
	17	0.59	0.78	29.7	72	1.2
	18	0.43	0.42	18.6	32	0.2
	19	0.48	0.41	12.4	30	1.9
	20	0.54	0.39	38.6	51	3.0
	21	1.06	0.29	20.5	26	0.0
	22	0.40	0.49	21.4	55	0.0
	23	0.46	0.70	36.2	47	2.2
6	24	0.38	0.37	24.8	157	0.4
7	25	0.71	0.96	12.1	113	7.6
8	26	1.08	1.25	24.3	646	1.2
9	27	6.13	3.94	15.1	301	8.1
	28	4.60	4.79	22.6	140	0.0
	29	3.31	2.46	21.4	132	0.0

[a] Contributions to total χ^2 between potencies.

[b] Crop-weight used as response.

DISTRIBUTION OF WEIGHTED LOG POTENCIES OF SECOND INTERNATIONAL STANDARD FOR PROLACTIN



Combination of results

The 29 estimates of log potency were slightly heterogeneous ($\chi^2 = 44.7$; $0.05 > P > 0.02$), the two largest contributions to χ^2 coming from assay 25 (which was the sole contribution of Laboratory 7) and assay 27 (the only test in which the rat was used as test animal). The heterogeneity is clearly due to interlaboratory variation, the variation within laboratories contributing an insignificant amount to the total χ^2 (Table 5).

The variation between estimates obtained by any particular laboratory was not sufficient to give a significant value for χ^2, although that for Laboratory 9, which included the test on rats, was approaching significance (Table 6).

The over-all weighted mean potency is 21.99 IU/mg with confidence limits ($P = 0.95$) of 20.34-23.78 IU/mg, i.e., 92.5%-108.1% of the potency. These limits are based on the total weight of 3332.

TABLE 5
PARTITION OF HETEROGENEITY, χ^2

	χ^2	Degrees of freedom	P
Between laboratories	25.9	8	0.001-0.01
Within laboratories	18.8	20	0.5-0.7
Total	44.7	28	0.02-0.05

Use of the heterogeneity factor (for variation between laboratories) as described by Emmens (1948) widens the limits to 19.11-25.31 IU/mg, i.e., 86.9%-115.1% of the potency.

ESTABLISHMENT OF THE SECOND INTERNATIONAL STANDARD FOR PROLACTIN

It is unfortunate that only 29 comparisons of the proposed standard with the First International Standard have been obtained for the purpose of assigning a potency to the former and that one-half of these, representing a quarter of the total statistical weight, came from a single laboratory. If all laboratories were in agreement this amount of information should be sufficient for the reliable estimation of a potency, but more comparisons would be required to decide which variables caused discrepancies between laboratories.

In the collaborative assay of the First International Standard (Emmens, 1939) it was found that in assays

TABLE 6
COMBINATION OF RESULTS WITHIN LABORATORIES

Lab. No.	No. of assays	Weighted mean potency (IU/mg)	Total weight	Consistency within laboratories χ^2	Consistency within laboratories P
1	2	17.3	165	0.5	0.3-0.5
2	1	19.0	152	—	—
3	4	18.9	384	1.9	0.5-0.7
4	2	24.0	284	0.6	0.3-0.5
5	14	27.5	858	11.9	0.5-0.7
6	1	24.8	157	—	—
7	1	12.1	113	—	—
8	1	24.3	646	—	—
9	3	18.1	573	3.9	0.1-0.2

where the local, intradermal method of injection was used the potencies were only one-tenth of those obtained when the preparations were administered systemically. In the present study the results of assays 28 and 29 are very close to the weighted mean obtained from all assays. The test in rats has, however, given a rather low and heavily weighted estimate of potency, but since the method has been used for just a single assay it is impossible to decide whether this is a chance result or a real difference in potency due to assay technique.

Bates & Riddle (1936) investigated the effects of using different routes of injection for prolactin on crop-weight and claimed that injection by the subcutaneous route was five times as effective as injection intramuscularly. The figure was deduced from direct comparison of the crop-weights of pigeons which had all been treated with the same preparation of prolactin. From the collaborative assay there is some suggestion that the potency of one preparation in terms of another may be lower when intramuscular injection is used. Laboratory 3 obtained higher potencies as the result of subcutaneous injection in comparison with their other assays in which the preparations were administered intramuscularly. The potencies estimated by Laboratories 1 and 2 are also at the lower end of the distribution. This conclusion is not supported by the results from Laboratory 4, where intramuscular injection was used, the resulting potencies occupying a central position in the distribution. It may be pertinent to note that Laboratories 1 and 3 consistently found that the slope of the log-dose/response lines for the proposed standard was less than that for the First Standard while Laboratory 4 produced log-dose/response lines of almost equal slope for the two preparations.

Dividing the dose into four or six injections appears to have no effect on the estimated potency. Laboratory 5 obtained potencies in the range 18.6-38.6 IU/mg when the dose was given in four parts and 12.4-37.8 IU/mg when the dose was divided into six. It does, however, seem that the slopes of the log-dose/response lines tend to be larger and more variable after six injections (range 0.45-0.94) than after four (range 0.42-0.49). Folley et al. (1940) observed that the slopes were steeper after six injections. Dr Bates reports (unpublished observations) that variability is much diminished (and a steeper slope obtained) by using older pigeons (5-8 years of age), injected intramuscularly for seven days.

More than half the total χ^2 for heterogeneity between laboratories comes from the two extreme potencies, estimated by Laboratories 5 and 7. There is no apparent reason for the low value which was found by Laboratory 7. At this laboratory the new standard, which is extremely hygroscopic, was evidently weighed before dilution, but the material was also weighed at Laboratories 2, 4 and 8 without any untoward result.

Some of the individual potencies obtained by Laboratory 5 are very high in comparison with those found by other laboratories, but they are mostly of low weight and the entire distribution of potencies for this laboratory forms a homogeneous set covering the whole range of values found by other workers.

It is concluded that there is insufficient evidence to select any particular assays as the cause of variation in the results and that the best available estimate of the potency of the proposed standard in terms of the First International Standard is the weighted mean of 22 IU/mg.

With the agreement of participants in the collaborative assay and in accordance with the authorization of the WHO Expert Committee on Biological Standardization (1957), the Second International Standard for Prolactin has therefore been established with a potency of 22 IU/mg.

The International Unit of Prolactin is thus defined as the activity contained in 0.04545 mg of the Second International Standard for Prolactin.

Annex

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RÉSUMÉ

En 1953 la provision de premier étalon international de prolactine commençait à s'épuiser et dans sa septième session, le Comité OMS d'experts de la Standardisation biologique a demandé au National Institute for Medical Research de faire le nécessaire pour le remplacer. Un lot de 50 g de prolactine purifiée provenant d'hypophyses de mouton a été obtenu en 1956. L'année suivante ce matériel a été dissous, réparti dans 2330 ampoules et desséché sous congélation. Chaque ampoule contenait 10 mg (± 5%) de poudre sèche. Après une seconde et complète dessiccation sur pentoxyde de phosphore les ampoules ont été emplies d'azote et scellées.

Suivant l'autorisation donnée par le treizième rapport du Comité OMS d'experts de la Standardisation biologique l'on a maintenant établi le deuxième étalon international de prolactine avec une puissance de 22 UI/mg. Ce chiffre résulte d'une série d'essais de la substance prévue comme deuxième étalon international par rapport au premier étalon international. Neuf laboratoires appartenant à cinq pays ont participé à cette étude et effectué 29 essais, 26 d'entre eux selon des variantes de la méthode du jabot de pigeon. Les limites de confiance ($P = 0,95$) de la puissance finale (22 UI/mg) sont d'environ ± 15%. L'unité internationale de prolactine est définie par l'activité contenue dans 0,04545 mg du second étalon international de prolactine.

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